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Similar Distribution of Gonadotropin Isohormones Is Maintained in the Pituitary Throughout Sexual Maturation in the Heifer

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A Similar Distribution of Gonadotropin Isohormones Is Maintained in the Pituitary Throughout Sexual Maturation in the Heifer[1]

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ABSTRACT

Our working hypotheses for this study were that 1) the profile of intrapituitary LH and FSH isoforms would be shifted toward acidic forms as sexual maturation progresses in the bovine female; and 2) concentration of 17β-estradiol (E_2) in circulation during sexual maturation would be a major factor modulating the percentage of the more acidic isoforms. In addition, the biological:immunoreactive (B:I) ratios of each isoform of LH were evaluated at selected stages of sexual maturation. Heifers (7 mo of age) were assigned to one of three treatment groups: 1) ovariectomized (OVX; n = 16); 2) OVX and administered E_2 (OVXE; n = 16); or 3) ovary-intact (INTACT; n = 14). Pituitaries were collected from heifers in each group at an estimated 120 days (prepubertal) of 25 days before puberty (peripubertal). A fourth group of 6 heifers remained intact (postpubertal INTACT) to determine time of puberty during the experimental period. Pituitaries of heifers assigned to the postpubertal INTACT group were collected during the follicular phase of the first or second estrous cycle postpuberty. Pituitaries were used for determination of relative amounts of gonadotropin isohormones. Tissue extracts of the pituitaries were chromatofocused on pH 10.5–4.0 gradients. The LH of all pituitaries resolved into thirteen isoforms that were designated isoforms A–L, and S, with isoform A the most basic form. Isoforms F and G (basic pH range) were the predominant isoforms of each chromatofocusing profile and comprised 50–60% of the immunoreactive LH. Isoforms J and K were the major isoforms eluting in the acidic pH range. Decreases in the acidic isoforms J and K were observed in heifers from the prepubertal and peripubertal OVX groups when compared to those observed in age-matched heifers from the INTACT of OVXE groups. Ovariectomy caused a concomitant increase in the more basic isoforms (B–E) in heifers from the OVX groups when compared to age-matched heifers from the INTACT or OVXE groups. The distributions of LH isoforms in all heifers of the INTACT groups (regardless of stage of sexual maturation) were similar. Isoform F had the greatest B:I ratio, whereas isoform A (most basic) and isoforms I, J, K, L, and S (most acidic) had the lowest biological potency. The isoforms of FSH were coded with Roman numerals beginning with the most basic form (I–IX). Heifers from the prepubertal and peripubertal OVX groups had lower relative amounts of the most acidic FSH isoform (IX) as compared to age-matched INTACT heifers. Heifers from the OVX group showed an increase in the relative amount of the most basic FSH isoform as compared to age-matched INTACT heifers.

In summary, removal of the ovary causes a change in the distribution of isoforms of both LH and FSH, and administration of physiological levels of E_2 to OVX heifers helps restore the acidic isoforms of the gonadotropins to levels seen in INTACT heifers. Our hypotheses were not supported in that there was no change in the distribution of LH or FSH isoforms occurred during sexual maturation in intact bovine females

INTRODUCTION

The influence of 17β-estradiol (E_2) in modulation of gonadotropin secretion varies as sexual maturation progresses in heifers. Ovariectomy of prepubertal heifers has been found to cause an increase in both mean concentration of LH and frequency of LH pulses [1–3]. Furthermore, active immunization of intact prepubertal heifers against E_2 results in an increase in concentrations of LH [4].

Ramirez and McCann [5] originally proposed the gonadostat hypothesis for endocrine control of the sexual maturation process. According to this hypothesis, a decrease in the sensitivity of the hypothalamic-pituitary axis to negative feedback of E_2 allows increased secretion of gonadotropins and onset of estrous cycles. Early in sexual development, the hypothalamic-pituitary axis is hypersensitive to the negative feedback effects of E_2, thereby reducing pulsatile secretion of gonadotropins. As puberty approaches in heifers [3, 6], the inhibitory effects of E_2 begin to abate, and pulsatile release of LH increases. This increased secretion of LH results in stimulation of ovarian follicles, ovulation, and initiation of estrous cycles.

Charged isomers or "isohormones" of gonadotropins are also affected by E_2. Distribution of isoforms of LH and FSH can be changed by administration of E_2. This results in an increased percentage of acidic forms [7–9]. Isoforms of gonadotropins not only differ in their isoelectric point but also in biological activities. Isoforms of ovine LH that are very basic or very acidic have the lowest bioactive: immunoreactive potency (B:I) ratios [9]. Stage of sexual matura-

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tion influences distribution of isoforms for FSH in developing rats [10, 11]. The objectives of the present study were to examine the distribution and biological activity of isoforms of gonadotropins in heifers and to determine the influence of E_2 on these isoforms during sexual maturation.

MATERIALS AND METHODS

Experimental Protocol and Collection of Pituitaries

The experimental protocol and manipulation of heifers used in this study are described in detail in the companion paper [12]. Briefly, pituitaries were collected from heifers that were ovariectomized (OVX), OVX and administered E_2 (OVXE), or maintained as INTACT controls at an estimated 120 days (prepubertal) and 25 days (peripubertal) before puberty as estimated from the pattern of pulsatile LH secretion. Pituitaries were also collected from postpubertal heifers during the follicular phase of their first and second estrous cycle. Immediately after removal, anterior and posterior lobes of each pituitary were separated, and the anterior lobe was hemisected. Pituitary tissues were stored at −70°C until extracted. The E_2-replacement therapy for peripubertal OVXE heifers yielded a mean circulating concentration of 6.7 ± 0.7 pg/ml (Mean ± pooled SEM) of serum, which was similar to that in peripubertal INTACT heifers (4.9 ± 0.7 pg/ml [12]). In prepubertal OVXE heifers, the replacement therapy yielded a mean E_2 concentration of 14.7 ± 0.7 pg/ml of serum, similar to the mean concentration in postpubertal INTACT heifers in the follicular phase of their estrous cycles (12.2 ± 0.7 pg/ml) but significantly greater than that in corresponding prepubertal INTACT heifers (2.9 ± 0.7 pg/ml [12]). Prepubertal and peripubertal OVX heifers had mean circulating concentrations of E_2 of 1.7 ± 0.7 and 2.2 ± 0.7 pg/ml of serum, respectively [12].

Tissue Extraction

Frozen pituitary tissue was homogenized in 150 mM NaCl buffered with 50 mM Tris, pH 7.4, containing 0.5% (vol/vol) Triton X-100, 5 mM Na_2EDTA, 1 mM phenylmethylsulphonyl fluoride, 0.5 mg/L leupeptin, and 200 U/ml aprotinin (1.0 ml/100 mg wet tissue weight) for 30 sec using a polytron homogenizer (Brinkman Instruments, Westbury, NY). Pituitary extracts were clarified by centrifugation at 100 000 × *g* for 1 h, aliquoted into 0.5-ml portions (equivalent to 50 mg tissue), and stored at −70°C until chromatofocused.

Chromatofocusing

Four randomly selected pituitary extracts from each treatment group were subjected to chromatofocusing on pH 10.5–4.0 gradients. A 1-ml aliquot (100 mg tissue equivalents) was desalted by flow dialysis against water, with use of membranes with a 6 000–8 000-M_r cutoff (Spectra/Por 1; Spectrum Medical Industries, Inc., Los Angeles, CA). Desalted extracts were supplemented with Pharmalyte 8–10.5 (pH 7.0) to 2% (vol/vol) as well as with 2 mg each cytochrome c and myoglobin. Samples were then applied to 0.7 × 26-cm columns (volume = 10 ml; Kontes, Vineland, NJ) of PBE-118 (Pharmacia/LKB Biotechnology Inc., Piscataway, NJ) equilibrated with 25 mM triethylamine (pH 11.0). The columns were first eluted (5.0 ml/h) with Pharmalyte 8–10.5 (Pharmacia/LKB Biotechnology Inc.) diluted 1:45 with distilled water and adjusted to a pH of 7.0 with 6 N HCl. Seventy-five 1.5-ml fractions were collected until a stable plateau near pH 7.0 was obtained. The elution buffer was then switched to Polybuffer 74-HCl (Pharmacia/LKB Biotechnology Inc.) diluted 1:8 with distilled water and adjusted to a pH of 4.0 with 6 N HCl. An additional sixty-five 1.5-ml fractions were collected to reach a stable lower limiting pH of 4.0. Proteins bound to the column at this lower limiting pH were eluted with 1.0 M NaCl and collected as an additional twenty 1.5-ml fractions. The samples were allowed to reach room temperature, and the pH of each fraction was determined by using a Ross electrode. The samples were then buffered by adding 0.15 ml 1.1 M Tris (pH 7.0). Columns were re-equilibrated between samples with at least 50 column volumes of triethylamine. All buffers contained 1% glycerol and were completely degassed before use. Recovery of immunoreactive LH and FSH from the columns averaged 62% and 68%, respectively.

RIAs

Concentrations of LH in pituitary extracts and chromatofocusing fractions were determined by RIA [3, 13]. Rabbit antiserum against ovine LH (TEA-RAoLH #35), highly purified iodinated ovine LH (LER-1056-C2), and NIH-LH-B7 as a standard were used in the assay. Intra- and interassay coefficients of variation were 2.7% and 8.2%. Concentrations of FSH in pituitary extracts and chromatofocusing fractions were determined by RIA [3, 14]. Rabbit antiserum against ovine FSH (JAD-RAoFSH #17-6,7,9) and highly purified ovine FSH (LER-1976-A2) were used as radiolabeled tracer and standard. Intra- and interassay coefficients of variation were 3.0% and 6.9%, respectively.

In Vitro Bioassay

Chromatofocusing fractions corresponding to each LH isoform were pooled and designated as isoforms A–L and S. The pooled fractions for each isoform were dialyzed for a minimum of 24 h against two changes of distilled water and lyophilized. The lyophilized isoforms were reconstituted with medium used in the mouse interstitial cell bioassay (Medium 199 [Hanks' salts] containing 25 mM HEPES, 0.5 mM 3-isobutyl-1-methylxanthine [MIX], and 0.1% BSA). The concentration of immunoreactive LH in the pool representing each peak was then quantified by RIA. The concentration of biologically active LH for each pool (isoform)

TABLE 1. Anterior pituitary weights and pituitary concentrations of gonadotropins.

Treatments*	Pituitary weight (mg)	Pituitary LH		Pituitary FSH	
		μg/mg tissue	μg/pituitary	μg/mg tissue	μg/pituitary
Prepubertal OVX	877.8^{c}	0.97^{c}	827.3^{c}	0.028^{b}	25.3^{b}
Peripubertal OVX	1,111.0^{bc}	0.87^{c}	968.3^{bc}	0.031^{b}	36.2^{b}
Prepubertal OVXE	992.3^{bc}	0.81^{c}	797.3^{c}	0.070^{a}	73.5^{a}
Peripubertal OVXE	1,547.8^{a}	0.96^{c}	1,496.3^{b}	0.037^{b}	58.2^{ab}
Prepubertal INTACT	839.8^{c}	1.80^{a}	1,327.9^{bc}	0.048^{ab}	44.3^{ab}
Peripubertal INTACT	1,181.0^{bc}	1.84^{a}	2,174.3^{a}	0.038^{b}	45.4^{ab}
Postpubertal INTACT	1,263.0^{ab}	1.35^{b}	1,573.8^{b}	0.040^{b}	49.8^{ab}
Pooled SEM	116.9	0.12	203.7	0.008	10.8

*Prepubertal: pituitaries collected approximately 120 days before attainment of puberty; peripubertal: pituitaries collected approximately 25 days before attainment of puberty; OVX: ovariectomized; OVXE: ovariectomized and administered E_2; INTACT: ovary-intact; postpubertal: pituitaries collected 40 h after administration of $PGF_{2\alpha}$ at first or second estrous cycle.

$^{a-c}$Means identified by different superscript letters within columns differ ($p < 0.05$).

was determined with the mouse interstitial cell-testosterone assay [15] using NIH-LH-B7 as the standard. An RIA (antisera, GDN #250) was used to determine the amount of testosterone produced [16, 17]. The intra- and interassay coefficients of variation for the testosterone assays were 6.7% and 16.2%, respectively. B:I ratios were calculated as the ratios of activity in the in vitro bioassay relative to the activity in the RIA. Distribution of biologically active LH among its isoforms was calculated for each pituitary extract by taking the concentration of LH in the tissue (μg LH/mg tissue) times the fraction of LH present as each isoform times the B:I ratio observed for that isoform (9).

Statistical Analysis

The effects of treatment on pituitary hormone characteristics were analyzed by one-way analysis of variance [18] utilizing the General Linear Models procedure. Differences in treatment means were established by Duncan's New Multiple Range test [18]. Percentage values were subjected to arc sine transformations (arc sine of the square root of the percentage) before analysis. The distribution of biologically active LH was analyzed after log transformation of the data. A probability of less than 0.05 was considered statistically significant.

RESULTS

Age at Tissue Collection

Mean estimated number of days to puberty for heifers in the peripubertal INTACT group was 4.3 ± 1.8 days [12]. In addition, the secretory pattern of LH detected immediately before the time tissues were collected in heifers from the peripubertal INTACT group was characteristic of the secretory pattern of LH pulses in heifers less than 25 days prepuberty [3, 19].

Pituitary Weight and Gonadotropin Content

Mean pituitary weights for heifers in the prepubertal groups were similar (Table 1). The mean pituitary weight for heifers in the peripubertal OVXE group was greater than those for heifers in the peripubertal OVX and peripubertal INTACT groups but similar to that for heifers in the postpubertal INTACT group. Mean pituitary weights for heifers from postpubertal INTACT, peripubertal INTACT, and OVX groups were similar. Mean pituitary concentrations of LH (μg/mg) were greater in heifers from prepubertal and peripubertal INTACT groups than in heifers from other treatment groups (Table 1). Heifers in the postpubertal INTACT group had an intermediate mean concentration of LH compared to heifers in the prepubertal and peripubertal INTACT and all OVX groups. A twofold increase in pituitary concentrations of LH was observed among heifers from the prepubertal and peripubertal INTACT groups compared to those from OVX or OVXE groups.

Total pituitary content of LH (Table 1) was greatest in heifers from the peripubertal INTACT group. Heifers from the postpubertal INTACT and peripubertal OVXE groups had greater total pituitary LH content than heifers from the prepubertal OVX or OVXE groups. The mean of total pituitary LH content for heifers from the prepubertal OVX or OVXE groups was similar to means for heifers in all other groups with the exception of heifers in the peripubertal INTACT group.

Pituitary concentrations of FSH (μg/mg) were greatest in heifers from the prepubertal OVXE group (Table 1). Total pituitary content of FSH was greatest in the heifers from the prepubertal OVXE group (Table 1). Generally heifers in the prepubertal and peripubertal OVX groups had the lowest content of FSH in the pituitary. Heifers in the INTACT and peripubertal OVXE groups had an intermediate content of pituitary FSH as compared to heifers in the prepubertal OVXE group.

LH Isoforms

Extracts of all anterior pituitaries resolved into thirteen isoforms of LH (Table 2) when chromatofocused over pH 10.5–4.0 gradients. The isoforms of LH were coded with letters A–L and S beginning with the most basic form. Twelve

TABLE 2. Distribution of bovine LH isoforms in anterior pituitary tissue.

Isohormone (Elution pH)	Treatments*							
	Prepubertal OVX	Peripubertal OVX	Prepubertal OVXE	Peripubertal OVXE	Prepubertal INTACT	Peripubertal INTACT	Postpubertal INTACT	Pooled SEM
A (9.82 ± 0.04)	0.10^{abc}**	0.15^{a}	0.06^{bcd}	0.15^{ab}	0.05^{cd}	0.03^{d}	0.04^{cd}	0.02
B (9.11 ± 0.01)	1.47^{a}	1.58^{a}	0.45^{b}	1.38^{a}	0.52^{b}	0.52^{b}	0.50^{b}	0.21
C (9.03 ± 0.01)	1.48^{a}	0.78^{ab}	0.55^{bc}	0.81^{ab}	0.33^{bc}	0.50^{bc}	0.18^{c}	0.24
D (8.95 ± 0.01)	4.85^{a}	3.61^{a}	1.49^{b}	1.69^{b}	2.10^{b}	2.27^{b}	2.06^{b}	0.44
E (8.89 ± 0.01)	12.33^{ab}	13.51^{a}	4.59^{d}	7.17^{c}	9.86^{bc}	9.61^{bc}	8.41^{c}	1.01
F (8.80 ± 0.01)	37.46^{ab}	41.41^{a}	32.48^{b}	41.57^{a}	42.34^{a}	42.51^{a}	43.22^{a}	2.01
G (8.66 ± 0.01)	21.72	22.35	24.12	23.53	23.38	23.09	25.57	1.41
H (7.29 ± 0.03)	3.56^{bcd}	3.28^{cd}	5.27^{a}	5.18^{ab}	4.03^{abcd}	4.55^{abc}	2.85^{d}	0.47
I (6.59 ± 0.02)	1.62	1.58	1.93	1.54	1.64	2.05	1.31	0.25
J (6.20 ± 0.01)	6.20^{b}	4.35^{c}	12.29^{a}	6.82^{b}	7.50^{b}	6.82^{b}	8.39^{b}	0.75
K (5.91 ± 0.01)	3.70^{bcd}	2.73^{d}	7.66^{a}	4.92^{b}	4.00^{bc}	3.55^{bcd}	3.42^{cd}	0.50
L (5.14 ± 0.04)	4.32^{b}	3.55^{bc}	7.04^{a}	3.84^{bc}	3.49^{bc}	3.24^{bc}	2.85^{c}	0.53
S (<4.0)	1.34^{b}	0.97^{b}	2.03^{a}	1.07^{b}	1.39^{b}	1.27^{b}	1.19^{b}	0.16

*Prepubertal: pituitaries collected approximately 120 days before attainment of puberty; peripubertal: pituitaries collected approximately 25 days before attainment of puberty; OVX: ovariectomized; OVXE: ovariectomized and administered E_2; INTACT: ovary-intact; postpubertal: pituitaries collected 40 h after administration of $PGF_{2\alpha}$ at first or second estrous cycle.

**Mean percentages for each isoform (n = 4).

$^{a-d}$Means identified by different superscript letters within rows differ ($p < 0.05$).

isoforms eluted in the separating pH range of the column and the thirteenth isoform was bound to the column at the lower limiting pH. The predominant isoforms eluted in the basic pH range were F and G, and these forms accounted for at least 55% of the immunoreactive LH in the pituitary. The predominant isoforms in the acidic elution pH range were J and K, and these forms accounted for approximately 10% of the immunoreactive LH in the pituitary.

Effects of Treatment on Distribution of LH Isoforms

The LH in pituitaries of OVX heifers generally consisted of higher percentages of the basic isoforms as well as lower percentages of some of the acidic isoforms, compared to LH in pituitaries of INTACT heifers at similar stages of sexual maturation. For example, prepubertal OVX heifers had higher percentages of isoforms B, C, and D than did prepubertal INTACT heifers, while peripubertal OVX heifers had higher percentages of isoforms B, D, and E as well as a lower percentage of isoform J than did corresponding peripubertal INTACT heifers (Table 2). Overall, ovariectomy induced a subtle shift in the distribution of LH toward basic isoforms.

The profiles of LH isoforms in pituitaries of heifers in the OVXE groups were altered as a result of circulating concentrations of E_2. For example, when concentrations of E_2 were similar in the peripubertal OVXE and INTACT groups, the pattern of LH isohormones in the peripubertal OVXE group was similar to that in the peripubertal INTACT group except for a higher percentage of isoform B in the OVXE group (Table 2). In contrast, administration of E_2 to prepubertal OVXE heifers yielded concentrations of E_2 that were significantly higher than those of heifers in the prepubertal INTACT group but similar to the follicular-phase concentrations observed in postpubertal INTACT heifers [12]. In the presence of these relatively high circulating concentrations of E_2, heifers from the prepubertal OVXE group had a much higher percentage of acidic forms of LH than did INTACT heifers (31% versus 17–18%, respectively). In particular, heifers from the prepubertal OVXE group had reduced percentages of isoforms E and F as well as significantly higher percentages of isoforms J, K, and L. Thus, circulating concentrations of E_2 corresponding to those observed in the follicular phase of the estrous cycle of postpubertal heifers caused a shift towards acidic isoforms of LH in prepubertal heifers.

The distributions of LH among its isoforms were similar in INTACT heifers at the three stages of sexual maturation (prepubertal, peripubertal, and postpubertal) (Table 2, Fig. 1). Even though the results obtained with OVX and OVXE heifers illustrate that the intracellular pattern of LH isoforms can be altered by endocrine signals, no major changes in the profile of LH isoforms were apparent in INTACT heifers during sexual maturation.

B:I Ratios of the LH Isoforms

The B:I ratios for each LH isoform present in pituitary extracts are illustrated in Table 3. The acidic LH isoforms (I, J, K, L, and S), as well as the most basic isoform A, had the lowest B:I ratios. Isoform F consistently exhibited the highest B:I ratio. Isoforms B, C, D, and E had B:I ratios intermediate to those of acidic isoforms and isoform F. For the most part, the B:I ratio for a given isoform of LH was independent of treatment. Minor differences as a result of treatments were observed for the three most acidic isoforms of LH (K, L, and S). Mean values for the distribution of biologically active LH for each treatment group are illustrated in Table 4. Clearly, isoforms F and G are predominant and constitute the vast majority of the biologically active hormone in the pituitary.

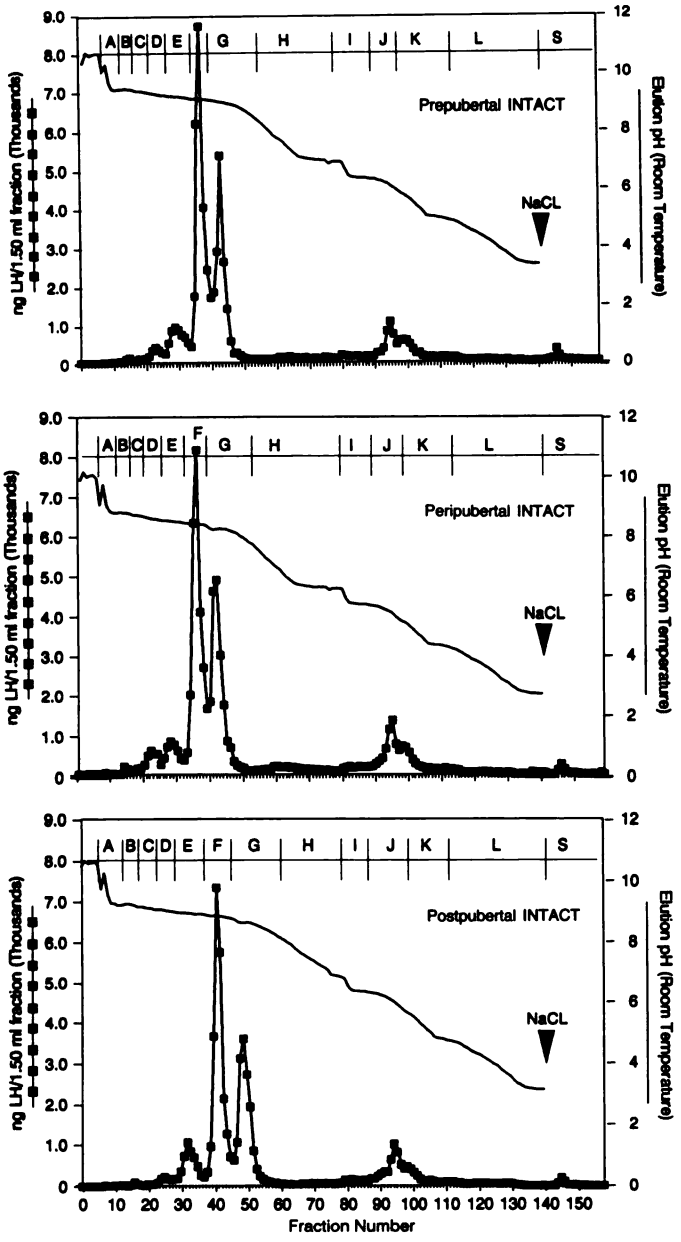

FIG. 1. Representative chromatofocusing profiles Isoform S was bound to the column and eluted with 1.0 M NaCl.

FSH Isoforms

The FSH in extracts of anterior pituitaries resolved into nine isoforms when chromatofocused on pH 10.5–4.0 gradients (Table 5) Isoforms of FSH were coded with Roman numerals I–IX, beginning with the most basic form. Seven isoforms eluted in the separating pH range of the column (I–VII), while isoforms VIII and IX were bound to the column at the lower limiting pH of 4.0. One isoform of FSH, I, eluted in the basic pH range (pH > 7.0), with all other isoforms of FSH eluting in the acidic pH range (pH 7.0–4.0). Isoform VIII characteristically eluted within one fraction after the elution buffer was changed to 1 M NaCl, whereas isoform IX eluted approximately eight fractions after application of 1 M NaCl.

Effects of Treatment on Patterns of FSH Isoforms

Distributions of isoforms I, III, VI, and IX were affected by treatment (Table 5). Heifers in the prepubertal and peripubertal OVX groups had greater percentages of isoform I (elution pH = 8.67) and lower percentages of isoform IX (elution pH < 3.8) than heifers in the corresponding INTACT groups. Therefore, ovariectomy shifted the distribution of FSH toward basic forms, as was the case with LH.

Heifers in the peripubertal OVXE group had a significantly greater percentage of isoform I and a significantly lower percentage of isoform IX than corresponding heifers of other groups. Thus, heifers from the peripubertal OVXE group exhibited a pattern of FSH isohormones more similar to patterns for OVX than for INTACT animals. In contrast, heifers from the prepubertal OVXE group had a higher percentage of isoform IX and lower percentage of isoforms I and III. Hence, prepubertal OVXE heifers exhibited a significant shift in the profile of FSH isohormones toward acidic forms, similar to that observed for LH.

The distributions of FSH among its isoforms were similar in INTACT heifers at the three stages of sexual maturation (prepubertal, peripubertal, and postpubertal) examined (Table 5, Fig. 2). The results obtained with OVX and OVXE heifers illustrate that the intracellular patterns of FSH isoforms, like those of LH isoforms, can be altered by endocrine signals; but as with LH isoforms, there do not appear to be major changes in the profile of FSH isoforms as sexual maturation progresses in the heifer.

DISCUSSION

The experimental protocol used in the present study allowed for collection of tissue at selected times during sexual maturation, specifically during prepuberty (an estimated 120 days prior to puberty), during peripuberty (an estimated 25 days prior to puberty), and postpuberty. These specific times were chosen on the basis of previous studies demonstrating that the prepubertal heifer is hypersensitive to negative feedback effects of E_2 and that sensitivity is reduced in the peripubertal heifer [2, 3, 12]. Tissues were also collected postpuberty during the follicular phase of the estrous cycle when E_2 has a positive feedback effect on LH secretion [20]. To examine the specific role of E_2, OVX heifers were administered E_2. Physiologic concentrations of E_2 were successfully achieved in the heifers from the peripubertal OVXE group, whereas levels of E_2 corresponding to concentrations found in the follicular phase of the estrous cycle were administered to heifers from the prepubertal OVXE group [12]. Levels of E_2 achieved in the latter group were higher than anticipated and were significantly greater than those observed in heifers from the prepubertal INTACT group. Patterns of LH secretion observed in heifers

TABLE 3. B:I ratios of LH isoforms in the anterior pituitary.

Isohormone (Elution pH)	Treatments*							
	Prepubertal OVX	Peripubertal OVX	Prepubertal OVXE	Peripubertal OVXE	Prepubertal INTACT	Peripubertal INTACT	Postpubertal INTACT	Pooled SEM
A (9.82 ± 0.04)	0.05**	0.04	0.05	0.02	0.01	0.10	0.06	0.02
B (9.11 ± 0.04)	0.28	0.25	0.09	0.15	0.13	0.29	0.18	0.06
C (9.03 ± 0.01)	0.13	0.15	0.13	0.07	0.13	0.18	0.09	0.03
D (8.95 ± 0.01)	0.20	0.15	0.12	0.09	0.14	0.19	0.17	0.05
E (8.89 ± 0.01)	0.19	0.19	0.11	0.11	0.20	0.19	0.15	0.03
F (8.80 ± 0.01)	0.51	0.32	0.27	0.23	0.32	0.37	0.26	0.05
G (8.66 ± 0.01)	0.31	0.17	0.23	0.10	0.19	0.23	0.23	0.06
H (7.29 ± 0.03)	0.10	0.12	0.04	0.06	0.12	0.10	0.08	0.03
I (6.59 ± 0.02)	0.05	0.04	0.02	0.02	0.05	0.04	0.03	0.01
J (6.20 ± 0.01)	0.09	0.08	0.04	0.04	0.10	0.08	0.06	0.02
K (5.91 ± 0.01)	0.07^{a}	0.08^{a}	0.03^{c}	0.04^{bc}	0.06^{ab}	0.08^{a}	0.07^{a}	0.01
L (5.14 ± 0.04)	0.09^{a}	0.10^{a}	0.04^{b}	0.05^{b}	0.08^{a}	0.09^{a}	0.09^{a}	0.01
S (<4.0)	0.07^{ab}	0.06^{ab}	0.03^{c}	0.04^{bc}	0.08^{a}	0.07^{ab}	0.07^{ab}	0.01

*Prepubertal: pituitaries collected approximately 120 days before attainment of puberty; peripubertal: pituitaries collected approximately 25 days before attainment of puberty; OVX: ovariectomized; OVXE: ovariectomized and administered E_2; INTACT: ovary-intact; postpubertal: pituitaries collected 40 h after administration of $PGF_{2\alpha}$ at first or second estrous cycle.

**Mean B:I ratio for each isoform (n = 3).

$^{a-c}$Means identified by different superscript letters within rows differ ($p < 0.05$).

of each group [12] corresponded to those anticipated and confirm that the endocrine manipulations had appropriate effects.

As anticipated, pituitary weights increased as sexual maturation progressed. Heifers from the OVX group had lower concentrations and contents of gonadotropins even though their peripheral concentrations of LH were elevated and characterized by frequent pulses [12]. Hence, cattle appear to respond in a manner similar to sheep, in which peripheral levels of gonadotropins are elevated but pituitary concentrations and contents of gonadotropins are reduced after gonadectomy [8, 9]. Heifers from the peripubertal OVXE group (E_2 replacement to physiological levels) had secretory LH patterns similar to those of postpubertal females during the follicular phase of their estrous cycle [12]. The relatively high amounts of LH and FSH in pituitaries of heifers from the postpubertal intact group may have resulted because of a buildup in stores of gonadotropin, which would have been released as the preovulatory surge of gonadotropin. In contrast, administration of follicular-phase levels of E_2 to prepubertal heifers yielded a relatively low pituitary content of LH but a high content of FSH. The low pituitary content of LH cannot be attributed to enhanced secretion [12]; instead, chronically elevated levels of E_2 appear to reduce synthesis of LH by inhibiting translation of the LH β gene [12]. In contrast, pituitary FSH was elevated in this group of heifers, suggesting differential regulation of the two gonadotropins by E_2 during prepuberty.

Gonadotropin isoforms were analyzed by chromatofocusing on pH 10.5–4.0 gradients. There were several rea-

TABLE 4. Distribution of biologically active LH in the anterior pituitary.

Isohormone (Elution pH)	Treatments*							
	Prepubertal OVX	Peripubertal OVX	Prepubertal OVXE	Peripubertal OVXE	Prepubertal INTACT	Peripubertal INTACT	Postpubertal INTACT	Pooled SEM
A (9.82 ± 0.04)	0.00**	0.03	0.00	0.00	0.00	0.00	0.00	0.01
B (9.11 ± 0.04)	4.30	2.30	0.26	1.83	1.50	2.30	1.06	0.95
C (9.03 ± 0.01)	1.46^{ab}	0.86^{ab}	0.46^{bc}	0.53^{abc}	0.83^{ab}	1.66^{a}	0.20^{c}	0.34
D (8.95 ± 0.01)	7.13^{a}	4.56^{a}	1.50^{b}	1.56^{ab}	6.03^{a}	7.06^{a}	5.16^{a}	1.79
E (8.89 ± 0.01)	18.56^{a}	19.80^{a}	3.80^{b}	6.66^{b}	38.63^{a}	30.43^{a}	17.83^{a}	7.08
F (8.80 ± 0.01)	182.50^{ab}	95.00^{bc}	55.80^{c}	90.83^{bc}	242.96^{a}	282.40^{a}	164.73^{abc}	40.45
G (8.66 ± 0.01)	71.66	26.0	34.40	24.60	80.80	99.76	79.43	22.67
H (7.29 ± 0.03)	3.60	2.33	1.70	2.73	8.40	9.20	3.30	2.34
I (6.59 ± 0.02)	0.63^{ab}	0.50^{ab}	0.20^{b}	0.26^{b}	1.53^{a}	1.46^{a}	0.63^{ab}	0.33
J (6.20 ± 0.01)	5.76	2.46	3.36	2.70	13.80	11.83	6.36	3.44
K (5.91 ± 0.01)	2.60^{abc}	1.56^{c}	1.83^{bc}	1.83^{bc}	4.73^{ab}	5.10^{a}	3.03^{abc}	0.87
L (5.14 ± 0.04)	3.76^{ab}	2.56^{b}	1.93^{b}	1.76^{b}	5.16^{a}	5.83^{a}	3.23^{ab}	1.05
S (<4.0)	0.80^{abc}	0.43^{bc}	0.36^{c}	0.40^{c}	2.16^{a}	1.73^{a}	1.03^{ab}	0.32

*Prepubertal: pituitaries collected approximately 120 days before attainment of puberty; peripubertal: pituitaries collected approximately 25 days before attainment of puberty; OVX: ovariectomized; OVXE: ovariectomized and administered E_2; INTACT: ovary-intact; Postpubertal: pituitaries collected 40 h after administration of $PGF_{2\alpha}$ at first or second estrous cycle.

**Mean ng of biologically active LH/mg pituitary (n = 3).

$^{a-c}$Means identified by different superscript letters within rows differ ($p < 0.05$).

TABLE 5. Distribution of bovine FSH isoforms in anterior pituitary tissue.

Isohormone (Elution pH)	Treatments*							
	Prepubertal OVX	Peripubertal OVX	Prepubertal OVXE	Peripubertal OVXE	Prepubertal INTACT	Peripubertal INTACT	Postpubertal INTACT	Pooled SEM
I (8.67 ± 0.02)	20.25^{ab}**	23.41^{a}	9.73^{d}	18.19^{b}	11.19^{cd}	12.86^{c}	10.94^{cd}	1.13
II (5.61 ± 0.01)	10.30	9.03	6.81	10.15	9.45	9.36	11.58	1.21
III (5.05 ± 0.01)	20.55^{a}	15.80^{bc}	12.32^{c}	18.01^{ab}	17.71^{ab}	14.59^{bc}	14.14^{bc}	1.30
IV (4.78 ± 0.01)	15.01	11.37	15.03	11.46	13.14	10.95	8.15	1.88
V (4.60 ± 0.01)	8.08	11.06	7.53	12.50	9.09	12.04	15.00	1.74
VI (4.36 ± 0.01)	6.27^{bc}	5.62^{bc}	11.87^{a}	8.83^{abc}	11.19^{a}	9.60^{abc}	10.88^{ab}	1.63
VII (4.12 ± 0.01)	8.18	11.44	13.32	8.92	11.04	12.78	11.93	1.25
VIII (3.83 ± 0.01)	2.58	1.74	3.57	1.62	4.08	2.76	2.75	0.87
IX (<3.80)	8.64^{d}	10.41^{cd}	19.71^{a}	9.41^{d}	13.10^{bc}	15.05^{b}	14.66^{b}	1.02

*Prepubertal: pituitaries collected approximately 120 days before attainment of puberty; peripubertal: pituitaries collected approximately 25 days before attainment of puberty; OVX: ovariectomized; OVXE: ovariectomized and administered E_2; INTACT: ovary-intact; postpubertal: pituitaries collected 40 h after administration of $PGF_{2\alpha}$ at first or second estrous cycle.

**Mean percentages for each isoform (n = 4).

$^{a-d}$Means identified by different superscript letters within rows differ ($p < 0.05$).

sons for extending the pH gradients below 7.0–4.0. First, this provided a means to characterize acidic forms of LH, which were of specific interest in the present experiment. Second, the isoforms of both LH and FSH could be characterized using a single chromatographic separation for each pituitary extract. The LH extracts of bovine pituitaries resolved into thirteen isoforms when chromatofocused on pH 10.5–4.0 gradients. The pattern of bovine LH isoforms observed in the basic portion of the gradient corresponds closely to results previously obtained for sheep [9, 21] and cattle [21, 22]. Similarly, bovine LH isoforms that eluted in the acidic range corresponded to the pattern observed when ovine pituitary extracts are chromatofocused on pH 7.0–4.0 gradients [23]. Hence, the pattern and distribution of LH isoforms are similar in ovine and bovine species.

In the present study, isoform F and, to a lesser extent, isoform G had the highest B:I ratios as appraised using an in vitro bioassay. Furthermore, the most basic isoform, A, as well as the LH isoforms that eluted in the acidic pH range (I through S) had relatively low B:I ratios. Similar results were previously reported for sheep [9] although the absolute magnitude of the B:I ratios was higher. Results in sheep were obtained using rat rather than mouse testicular cells, and hence the species from which the cells were obtained might contribute to differences in magnitude. More likely, the lower B:I ratios observed here can be attributed to the standard utilized. Results for the sheep were obtained using highly purified LH (oLH-DNW-MES-1-171D), whereas the less pure NIH-LH-B7 served as the reference standard in the present study. Irrespective of differences in absolute values, the pattern of B:I ratios observed in cattle correspond closely to those observed in sheep. Nonetheless, they are distinct from those observed in rats [24–26] and humans [27]. In these species, acidic forms of LH have relatively low B:I ratios; but isoforms with the most basic isoelectric points have the highest B:I ratios, and B:I ratios decrease in relation to apparent isoelectric points.

More than 55% of the LH in each pituitary extract eluted as isoforms F and G. In addition, these forms have the highest B:I ratios. Evaluation of the distribution of biologically active LH that takes both of these factors into consideration strongly suggests that isoforms F and G are the predominant bioactive forms of LH in the pituitary. In recent studies, the relationship between intracellular and secreted isoforms of LH has been examined [22]. The LH released by pituitary slices perifused in vitro in the presence or absence of LHRH contains higher percentages of F and G than are found in the corresponding tissue. Hence, the weight of the evidence suggests that LH isoforms F and G are likely to be the primary components exerting biological actions of LH in vivo. In the present study, B:I ratios were obtained with an in vitro procedure that effectively assesses actions at a target tissue but does not take circulatory survival into consideration. Thus, this general conclusion could be subject to change as information about circulatory survival and in vivo potencies of specific LH isoforms becomes available.

The FSH in pituitary extracts resolved into nine isoforms when chromatofocused on pH 10.5–4.0 gradients. Seven isoforms eluted in the separating pH range of the column, and two were bound to the column at the lower limiting pH. The first of these, isoform VIII, was not tightly bound and eluted immediately after 1 M NaCl was applied to the column. It is possible that this form would have eluted at the lower limiting pH of 4.0 had the plateau been extended. Nonetheless, some FSH, here called isoform IX, was tightly bound to the column and eluted as the 1 M NaCl front reached the bottom of the column. It is possible that isoform IX contains multiple components that could be resolved by extending the pH gradient further into the acidic range or by alternative procedures such as ion-exchange chromatography.

Analysis of isoforms of ovine FSH by chromatofocusing on pH 7.0–4.0 gradients also yields nine isoforms [8]. However, the most basic ovine FSH isoform elutes as a flow-

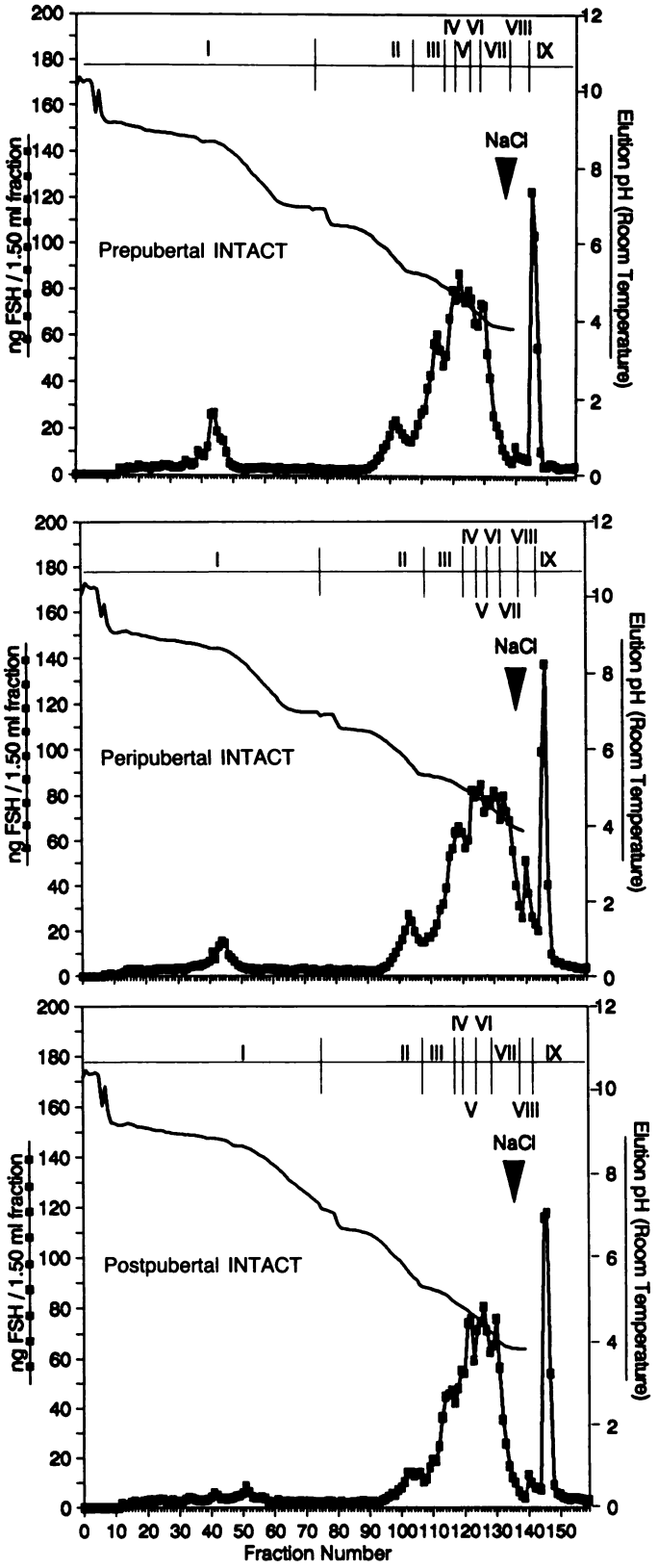

FIG. 2. Representative chromatofocusing profiles for the distribution of bovine FSH isoforms in prepubertal, peripubertal, and postpubertal INTACT heifers. Isoforms of FSH are coded with Roman numerals I–IX (beginning with the most basic form). Isoforms VIII and IX were bound to the column and eluted with 1.0 M NaCl.

through peak indicating an elution pH > 7.0. Interestingly, the most basic isoform of bovine FSH observed in the present study eluted at a pH similar to that of LH isoform G (mid-alkaline elution pH of 8.66). Because bovine FSH isoform I was the only one to elute in the basic pH range, we were concerned that our primary FSH antibody might selectively react with LH isoform G. In a subsequent experiment, we confirmed that FSH isoform I was similarly detected by the assay used here and the RIA used by Keel and Schanbacher [8], which employs reagents from the NIH Pituitary Hormone Distribution Program (Baltimore, MD). Thus, we believe that isoform I is indeed a somewhat distinctive form of bovine FSH. The structural determinants that result in its elution in the basic pH range remain to be elucidated. Moreover, the bioactivity of FSH isoforms in both cattle and sheep remain to be established.

Previous results have suggested that castration of sheep shifts the distribution of intrapituitary LH toward basic forms [9]. Cattle appear similar to sheep in that the profile of both LH and FSH isoforms was shifted toward basic forms as a result of ovariectomy. Several mechanisms that lead to this pattern can be envisioned. One possible mechanism is that reduced negative feedback of gonadal substances affects gonadotropin synthesis by altering posttranslational processing. Alternatively, enhanced release may contribute to the observed profile of intrapituitary isoforms of LH and FSH. Recent studies demonstrated that the pattern of LH isohormones released in vitro generally reflects intrapituitary stores, but that the released LH contains lower percentages of basic forms (specifically A, B, and C) and increased percentages of the mid-alkaline LH isoforms (specifically F and G [22]). The extremely basic forms have been proposed to represent intracellular processing intermediates [22]. Enhanced LH synthesis in ovariectomized heifers, as evidenced by relatively high LH β mRNA levels [12], could contribute to increased percentages of basic forms. Alternatively, enhanced release of mid-alkaline LH isoforms F and G could result in a pattern of intracellular LH isoforms that consists of a higher percentage of basic components. Further experimentation will be required to delineate whether one or both of these mechanisms contribute to increased percentages of basic gonadotropins in gonadectomized animals.

Previous results demonstrated that estrogens selectively increase the percentage of acidic LH [9] and FSH [8] isoforms in sheep, and acidic FSH isoforms in hamsters [7]. Data from heifers in the prepubertal OVXE group provided valuable information even though higher than physiological concentrations of E_2 were present. Elevated levels of E_2 clearly shifted the distribution of LH and FSH isoforms toward acidic components. These results illustrate that the pattern of gonadotropin isohormones can be modulated by E_2, particularly in prepubertal heifers. Interestingly, postpubertal heifers in the INTACT group that had circulating concentrations of E_2 similar to those of heifers in the prepu-

bertal OVXE group did not exhibit a shift in the distribution of gonadotropin isoforms toward acidic components.

Although results with OVX and OVXE heifers illustrate endocrine modulation of the profile of gonadotropin isohormones, the distribution of neither LH nor FSH among its isoforms was altered during sexual maturation in intact heifers. It was recently observed that ewes have similar distributions of intrapituitary isohormones during the follicular and luteal phases of their estrous cycles (Zalesky, D.D., Nett, T.M., Grotjan, H.E., unpublished results). One possible explanation is that the profile of gonadotropin isoforms is subject to minimal endocrine regulation during development of female cattle. Alternatively, hypothalamic and gonadal inputs into the pituitary, which are known to change during sexual maturation, may interact to maintain a fixed profile of gonadotropins during development.

In summary, removal of the ovary and thus the primary endogenous source of E_2 from heifers before puberty shifted the distribution of LH and FSH isoforms toward basic components. Administration of physiological levels of E_2 to ovariectomized heifers yielded a pattern of LH and FSH isohormones similar to that in intact animals, primarily by restoring the percentage of gonadotropins present as acidic isoforms. Ovariectomized heifers administered E_2 had higher percentages of acidic gonadotropin isoforms. Thus, it appears that E_2 acts to maintain the percentage of acidic gonadotropin isoforms during sexual maturation in the heifer. Nonetheless, the primary new information gained from this study is that distribution of isoforms for gonadotropins does not change significantly during sexual maturation in heifers.

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